

Progressive accumulation of the abnormal conformer of the prion protein and spongiform encephalopathy in the obex of nonsymptomatic and symptomatic Rocky Mountain elk (*Cervus elaphus nelsoni*) with chronic wasting disease

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Abstract. The purpose of our study was to describe the progressive accumulation of the abnormal conformer of the prion protein (PrP^{CWD}) and spongiform degeneration in a single section of brain stem in Rocky Mountain elk (*Cervus elaphus nelsoni*) with chronic wasting disease (CWD). A section of obex from 85 CWD-positive elk was scored using the presence and abundance of PrP^{CWD} immunoreactivity and spongiform degeneration in 10 nuclear regions and the presence and abundance of PrP^{CWD} in 10 axonal tracts, the subependymal area of the fourth ventricle, and the thin subpial astrocytic layer (glial limitans). Data was placed in a formula to generate an overall obex score. Data suggests that PrP^{CWD} immunoreactivity and spongiform degeneration has a unique and relatively consistent pattern of progression throughout a section of obex. This scoring technique utilizing a single section of obex may prove useful in future work for estimating the presence and abundance of PrP^{CWD} in peripheral tissues and the nervous system in elk with CWD.

Key words: Chronic wasting disease; elk, obex; prion protein; spongiform encephalopathy.

Introduction

Chronic wasting disease (CWD), a transmissible spongiform encephalopathy, has been reported in captive and free-ranging cervids.^{1,21,25,26} It is generally accepted that aberrantly folded prion protein (PrP) is associated with CWD and that this abnormal conformation can be detected with immunohistochemistry.²² The degree of PrP^{CWD} immunoreactivity (IR) and spongiform degeneration in the brain is thought to correlate with the duration of disease. It is speculated that the abundance of detectable PrP^{CWD} IR and spongiform degeneration spreads and increases in the brain with time and disease progression. However, this relationship has not been clearly established in Rocky Mountain elk (*Cervus elaphus nelsoni*) as suggested in free-ranging²¹ and experimentally infected mule deer (*Odocoileus hemionus*)² and captive white-tailed deer (*Odocoileus virginianus*).¹¹ We hypothesize that PrP^{CWD} spreads throughout the regions in the obex of the brain stem at a rate that may correspond with the movement of PrP^{CWD} through the nervous system and peripheral tissues. Herein we report the description of a detailed scoring technique, using a single section of the brain stem at the level of the obex, in elk with naturally occurring CWD.

This scoring technique may be useful for gaining insight into the spread of PrP^{CWD} and the pathogenesis of CWD in Rocky Mountain elk.

Materials and methods

Postmortem samples of brain stem, retropharyngeal lymph node, and palatine tonsil were obtained from ~300 free-ranging and 15,000 captive adult Rocky Mountain elk and were examined for the presence of PrP^{CWD} IR using

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immunohistochemistry.²² The free-ranging elk originated from northern Colorado and the captive elk originated from multiple herds throughout the Rocky Mountain region of the United States and Canada. Nearly all of these elk were euthanized by personnel affiliated with various state or federal wildlife agencies or by owners of the elk ranches, but a few were found dead. The elk that were found dead were all captive and had been observed for clinical signs before they died. Clinical signs included behavior changes, agitation, mild to moderate aggression especially when cornered or run through a chute, weight loss, excessive salivation, grinding of the teeth, and emaciation. Sex and age were recorded for many of the elk. Ages of elk were estimated by counting cemental rings of the first incisor teeth.¹² If fresh tissues were available, the *PRNP* genotype was determined.¹⁶

Brain stems, retropharyngeal lymph nodes, and palatine tonsils were fixed in 10% neutral buffered formalin for 1 week. A section of obex (brain stem at the level where the fourth ventricle converges into the central canal of the spinal cord and contains the dorsal motor nucleus of the vagus nerve), medial retropharyngeal lymph node, and palatine tonsil from each elk were trimmed, placed in plastic cassettes, and allowed to fix in fresh formalin for another 2–3 days prior to processing. Cassettes were processed overnight using conventional methods in an automated tissue processor as previously reported.²²

Sections of brain stem were cut at 5 μ m, and tissue ribbons were mounted on positively charged glass slides^a and dried in a 65°C oven overnight. Slides were deparaffinized, placed in 99% formic acid^b for 5 min at room temperature, and then rinsed in running tap water for 5 min. Slides were then autoclaved for 20 min at 121°C in citrated buffer,^c cooled, and transferred into Tris buffer.^d Staining was performed with an automated immunostainer at 37°C using an alkaline phosphatase red kit with amplification.^d Tissues sections were immunolabeled with an anti-prion antibody kit (clone 99/97.6.1^d), which uses a mouse monoclonal antibody derived from ascites for use on the aforementioned automated slide staining system.^d Specific antibody activity is ~8 μ g/mL in a Tris-based buffer. Monoclonal antibody F99/97.6.1 binds an epitope at residues 220–225, near the carboxyl terminus of the processed prion protein.¹⁶ This monoclonal antibody was incubated on slides for 32 min at 37°C. The slides were rinsed and amplifier A (rabbit anti-mouse IgG heavy and light chains) was applied and incubated for 8 min at 37°C while amplifier B was run using an empty dispenser. The slides were rinsed, and the universal biotinylated secondary antibody was applied and incubated for 8 min at 37°C. Next, the slides were washed, and the streptavidin–alkaline phosphatase conjugate was applied and incubated for 12 min at 37°C with mixing. Fast Red A^d and naphthol^d were applied and incubated for 8 min at 37°C. Next, Fast Red B^d was applied to the slide and incubated for 8 min at 37°C. Slides were counterstained with hematoxylin for 4 min at 37°C. Positive and negative control slides

containing lymphoid tissues and brain were stained with each run. When used following the immunohistochemistry procedures as described by National Veterinary Services Laboratories (NVSL), the commercially available anti-prion antibody (clone 99/97.6.1^d) kit will detect the prion protein in brain and lymphoid tissues in elk and deer with CWD. Routine hematoxylin and eosin–stained sections were examined for spongiform degeneration in each case.¹⁹

A polyclonal antibody specific to glial fibrillary acidic protein (GFAP), an astrocytic-specific marker, was used on sections of obex from 2 elk from each score category. The GFAP stain was performed using a commercial kit^d with no pretreatment. This polyclonal antibody was incubated on slides for 32 min at 37°C on an automated immunostainer with a basic alkaline phosphatase red kit.^d

A single section of obex from each CWD-positive elk was scored (raw score) by evaluation of the presence and abundance of PrP^{CWD} IR and spongiform degeneration in 10 nuclear regions and for the presence and abundance of PrP^{CWD} IR in 10 axonal tracts, the subependymal area of the fourth ventricle, and the thin subpial astrocytic layer (glial limitans). PrP^{CWD} IR was characterized by coarse granular red chromogen staining throughout these regions. This evaluation was done as follows: on low power (2 \times), and 10 nuclear areas of the obex including the dorsal motor nucleus of the vagus nerve (V), nucleus solitarius (S), area postrema (P), cuneate nucleus (C), nucleus of the spinal tract of the trigeminal nerve (T), nucleus ambiguus (A), reticular formation and nucleus (R), olive nucleus (O), medial raphe (MR), and the hypoglossal nucleus (H) were examined for presence of PrP^{CWD} IR.²⁷ Next, the abundance of PrP^{CWD} IR within each nucleus at 3 locations (intraneuronal, perineuronal, and within the neuropil) was evaluated subjectively and given a numeric score: 0 = not detected, 0.5 = trace, 1 = mild, 2 = moderate, and 3 = heavy (Figs. 1, 2). Lesions were bilaterally systematic, both sides were examined, and the average was recorded. Beaded linear and fine granular patterns, plaque formations of IR, and bright red granular material within Virchow–Robinson spaces were noted, but were not included in the scoring. Detectable PrP^{CWD} IR also was evaluated in the axonal tracts: solitary tract (ST), radix of the vagus nerve (RV), axonal tracts associated with the area postrema (TP), medial longitudinal fasciculus (MF), tectospinal tracts (TS), medial lemniscus (ML), pyramidal tracts (PT), ventral spinocerebellar tracts (VS), dorsal spinocerebellar tracts (DS), and spinal tract of the trigeminal nerve (SF). Detectable PrP^{CWD} IR in the glia limitans (GL) and in the loose tissue of the subependymal layer (SE) of the fourth ventricle was recorded with the axonal tracts. The abundance of PrP^{CWD} IR within each of the axonal tracts and the GL and SE was scored subjectively and given a numeric score: 0 = not detected; 0.5 = trace; 1 = mild; 2 = moderate; and 3 = heavy (Figs. 3, 4).

Routine hematoxylin and eosin–stained sections were examined for spongiform degeneration in each case. The degree of spongiform degeneration including the degree of

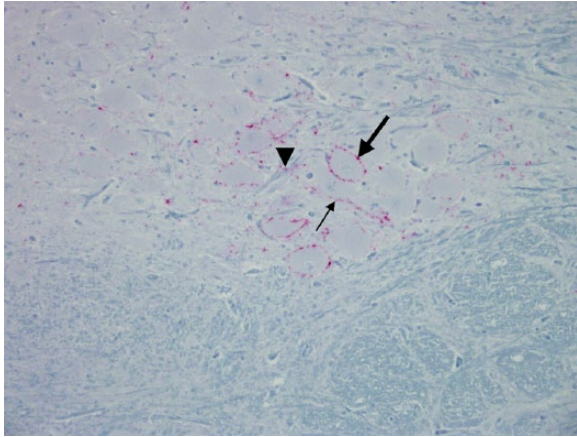


Figure 1. Dorsal motor nucleus of the vagus nerve; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 1. The grading for the perineuronal PrP^{CWD} immunoreactivity (IR) would be a 1 because only a few neurons are affected (thick arrow). The grade for the PrP^{CWD} IR within the neuropil also would be a 1 (arrowhead). Note the beaded linear pattern of PrP^{CWD} IR located within the neuropil that appears to be associated with an axon (thin arrow). Note the absence of intracytoplasmic PrP^{CWD} IR within the neurons. Immunohistochemical staining: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain.

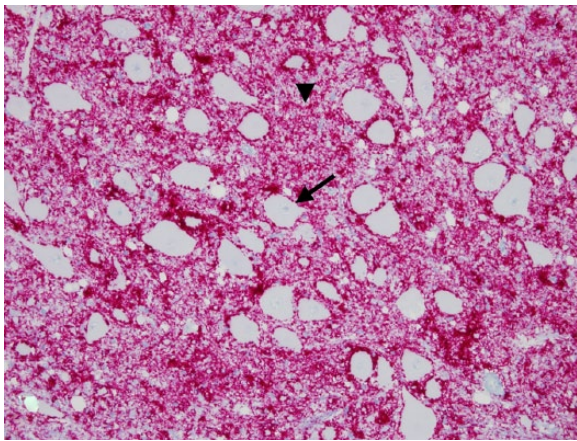


Figure 2. Dorsal motor nucleus of the vagus nerve; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 9. Note the extensive amount of PrP^{CWD} immunoreactivity (IR) located on the outer surface of neurons—numerical score of 3 (arrow). Note the extensive amount of PrP^{CWD} IR within the neuropil—numerical score of 3 (arrowhead). Note the absence of intracytoplasmic PrP^{CWD} IR within the neurons. Immunohistochemical staining: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain.

vacuolation within the neuropil, intraneuronal vacuolation, and neuronal necrosis was evaluated and recorded for each nucleus. Criteria used for scoring spongiform degeneration were the following: vacuolation of neuropil: 0 = not detected; 1 = <15 vacuoles; 2 = >15 but <35 vacuoles; and 3 = >35.

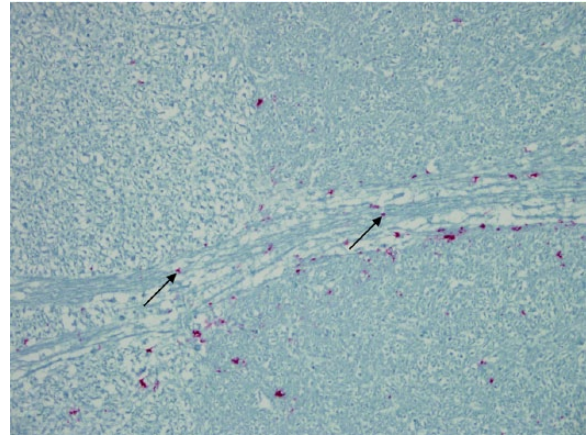


Figure 3. Axonal tract of the radix of the vagus nucleus; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 4. Note the minimal degree of PrP^{CWD} immunoreactivity (IR) located on the outer surface of axons (arrows). This would be a PrP^{CWD} IR abundance score of 1. Immunohistochemical staining: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain.

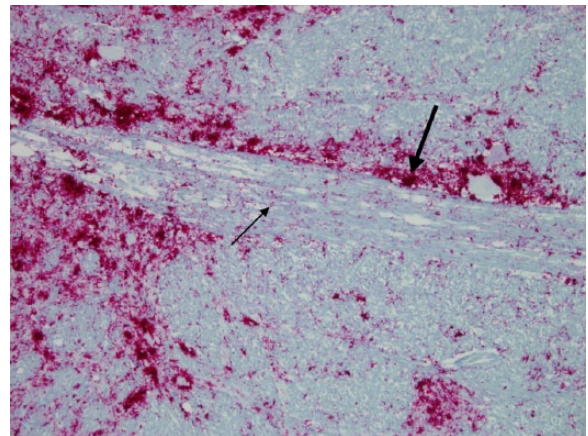


Figure 4. Axonal tract of the radix of the vagus nucleus; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 8. Note the abundance of PrP^{CWD} immunoreactivity (IR) located on the outer surface of axons (thin arrow). This would be an abundance score of 3. Also notice the extensive accumulation of PrP^{CWD} IR around the axonal tract (thick arrow). Immunohistochemical staining: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain.

Intraneuronal vacuolation was scored by counting the number of neurons containing vacuoles: 1 = 1–2 neurons; 2 = 3–4 neurons; 3 = 5–6 neurons; and 4 = ≥7 neurons. Neuronal necrosis was scored as follows: 1 = 1–2 necrotic neurons; 2 = 3–4; 3 = 5–6; and 4 = ≥7 (Figs. 5, 6). Criteria used for evaluating PrP^{CWD} IR and spongiform degeneration in our study was similar to that found in the literature describing immunohistochemical labeling and spongiform degeneration for profiling lesions in domestic sheep with naturally occurring and

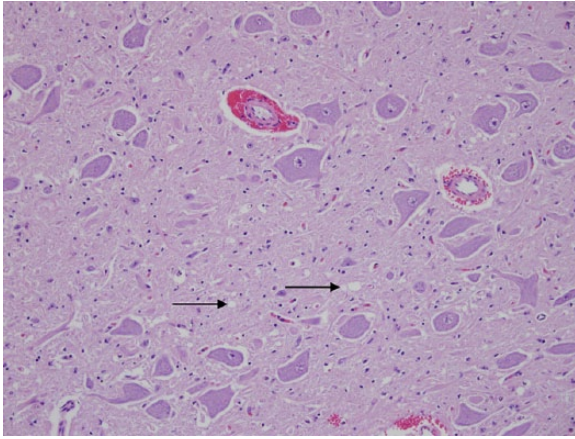


Figure 5. Dorsal motor nucleus of the vagus nerve; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 4. The degree of spongiform degeneration within the neuropil was mild (arrows). The neuropil vacuolation has a numeric score of 1; vacuolation of neurons was 0; and neuronal necrosis was 0. Hematoxylin and eosin.

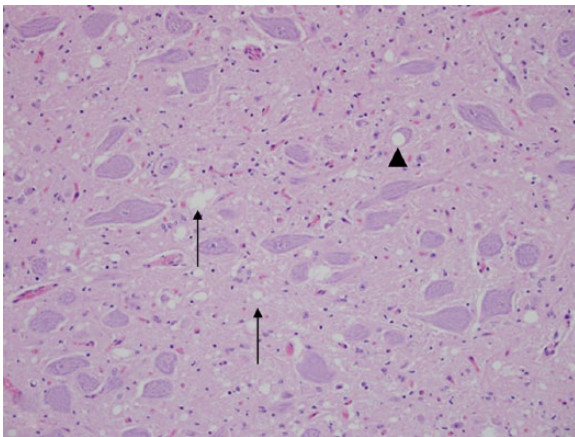


Figure 6. Dorsal motor nucleus of vagus nerve; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 6. The degree of spongiform degeneration within the neuropil was considered to be moderate, with a numeric score of 2 (arrows); vacuolation of neurons was a numeric value of 1 (arrowhead); and neuronal necrosis was 0. Hematoxylin and eosin.

experimental scrapie and bovine spongiform encephalopathy (BSE).^{3,4,7-9,13-15,24} Raw scores were recorded on a data sheet (Table 1). Following evaluation of these regions, the values were placed in a formula: $A + B + C + D + E + F = \text{raw obex score}$.

A = total number of nuclei with detectable PrP^{CWD} IR.
 B = average abundance of PrP^{CWD} IR in nuclei. Add the 3 (intraneuronal, perineuronal, and neuropil) numerical scores for all 10 nuclear locations then divide by 10.
 C = total number of axonal tracts with detectable PrP^{CWD} IR including the GL and the SE.

D = average abundance of PrP^{CWD} IR in the 10 axonal tracts including the GL and the SE. Add these abundance scores for each of the 12 locations and divide by 10.

E = total number of nuclei with spongiform degeneration.

F = average severity of spongiform degeneration. Add the numerical score for the 3 locations of spongiform degeneration for all 10 nuclear areas then divide by 10.

These 6 numbers are now added together ($A + B + C + D + E + F = \text{raw obex score}$; e.g., $10 + \{30/10\} + 12 + \{40/10\} + 10 + \{30/10\} = 42$, which indicates an elk in the terminal stages of CWD) to obtain the raw obex score. To simplify the scoring system, raw scores were grouped into 1 of 11 categories (Table 2).

Results

Of the elk that were examined for the presence of PrP^{CWD} IR, 323 were found to be positive (26 free-ranging and 297 captive). However, not all of the samples were suitable for use in our study. Suitable samples were those in which the section of obex contained all of the nuclear and axonal regions, and had minimal autolysis. Fifty-nine captive and 26 free-ranging elk were found to have samples meeting these requirements. Out of the 85 CWD-positive elk, 4 had PrP^{CWD} IR in lymphoid tissues, but not in the obex. These elk were included in our study and were given an obex score of 0. Two captive elk had PrP^{CWD} IR in the dorsal motor nucleus of the vagus nerve of the obex, but not in sections of retropharyngeal lymph nodes and palatine tonsil and were included in the study. The elk that did not have detectable PrP^{CWD} IR in any of the examined tissues were considered to be free of CWD. Even though PrP^{CWD} IR was not detected in any of these tissues from the “negative elk,” it does not totally rule out the presence of the CWD agent within these animals, it only indicates that the PrP^{CWD} prion was undetectable with our immunohistochemical technique within these specific tissues.

If fresh tissues were available, the *PRNP* genotype was determined.¹⁶ The 2 most common *PRNP* genotypes (132MM and 132ML) that have been reported in elk were found in our study.^{16,18} Genotype was determined in 49 of the elk (42 out of 49 (86%) 132MM; 6 out of 49 (12%) 132ML; and 1 (2%) 132LL; Table 3).

The sex was recorded in 57 of the positive elk and included 39 (69%) females and 18 (32%) males. Sixty-four of the 85 (75%) positive elk were non-clinical and presented no observable signs; whereas 21 (25%) showed typical clinical signs of CWD at the time of euthanasia or were observed with clinical signs prior to death. The ages were estimated in 43 elk and ranged from 1.5 to 16 years. Thirty-one of these 43 (70%) elk were ≤ 5 years of age.

Clinical signs were not observed in any of the elk with an obex score of ≤ 8 , and all of these elk were euthanized. Of the 14 elk with an obex score of 9, 7 showed clinical

Table 1. Data sheet used to record abundance of PrP^{CWD} immunoreactivity (IR) in nuclear regions and axonal tracts and severity of spongiform degeneration within nuclear regions from a single section of obex at the level of the dorsal motor nucleus of the vagus nerve.

RAW OBEX SCORING SHEET								
Elk number: _____		Obex score: _____						
Nuclei	PrP ^{CWD} IR ^a				Spongiform degeneration			
	N/D ^b	IN ^c	PN ^d	N ^e	N/D ^f	VN ^g	IN ^h	NN ⁱ
Dorsal motor nucleus vagus								
Area postrema								
Nucleus solitarius								
Cuneate nucleus								
N. spinal tract of trigeminal								
Nucleus ambiguus								
Reticular formation/nucleus								
Olive nucleus								
Medial raphe								
Hypoglossal nucleus								
Total								
Axonal tracts	N/D ^j	Abundance (1–3) ^k						
Solitary tract								
Radix of the vagus nerve								
Med longitudinal fasciculus								
Tectospinal tract								
Medial lemniscus								
Pyramidal tract								
Vent spinocerebellar tract								
Dorsal spinocerebellar tract								
Spinal tract, fifth nerve								
Tracts over area postrema								
Glial limitans								
Subependyma, fourth ventricle								
Total								

^a Immunohistochemical reactivity associated with the abnormal isoform associated with chronic wasting disease.

^b PrP^{CWD} IR: not detected (N) or detected (D).

^c PrP^{CWD} IR: intraneuronal staining (IN; 1 = mild, 2 = moderate, 3 = heavy).

^d PrP^{CWD} IR: perineuronal (PN; 1 = mild, 2 = moderate, 3 = heavy).

^e PrP^{CWD} IR: prion in neuropil (N; 1 = mild, 2 = moderate, 3 = heavy).

^f Spongiform degeneration: not detected (N) or detected (D).

^g Spongiform degeneration: vacuolation of neuropil (VN; 1 = mild, 2 = moderate, 3 = heavy).

^h Spongiform degeneration: intraneuronal vacuolation (IN; 1 = 1–2 neurons, 2 = 3–4, 3 = 5–6, 4 = ≥7).

ⁱ Spongiform degeneration: neuronal necrosis (NN; 1 = 1–2 neurons, 2 = 3–4, 3 = 5–6, 4 = ≥7).

^j PrP^{CWD} IR: not detected (N) or detected (D).

^k PrP^{CWD} IR: abundance (1 = mild, 2 = moderate, 3 = heavy).

signs and, of these 14 elk, all were euthanized except for 2. Of the 17 elk with an obex score of 10, clinical signs were reported in 11. Of these elk, 10 were euthanized and 7 were found dead.

Description of obex scores

Eleven categories (0–10) or obex scores were formulated based on grouping of raw scores in elk that were positive for CWD. The following is a brief description of the highlights

of the PrP^{CWD} IR and spongiform degeneration in these categories. The pattern of detectable PrP^{CWD} IR in the nuclei and axonal tracts and the severity of spongiform degeneration were progressive and similar in all 3 *PRNP* genotypes associated with the particular obex score (Table 4).

Obex score = 0 (raw score 0). Four elk had PrP^{CWD} IR in the medial retropharyngeal lymph node, but PrP^{CWD} IR was not detected in the palatine tonsil or 5 different levels of the V.

Table 2. Conversion chart for raw obex scores to final obex scores within a section of obex in Rocky mountain elk (*Cervus elaphus nelsoni*) with chronic wasting disease.

Raw obex score	Obex score
0	0
1 but ≤ 2	1
>2 but ≤ 5	2
>5 but ≤ 10	3
>10 but ≤ 15	4
>15 but ≤ 20	5
>20 but ≤ 25	6
>25 but ≤ 30	7
>30 but ≤ 35	8
>35 but ≤ 40	9
≥ 40	10

Table 3. Summary of obex scores in the 3 different *PRNP* genotypes from 26 free-ranging and 59 ranch-raised Rocky Mountain elk (*Cervus elaphus nelsoni*) with chronic wasting disease.

Obex score	Free-ranging elk				Ranch-raised elk			Total
	MM*	ML†	LL‡	ND§	MM	ML	ND	
0	1	2	0	0	1	0	0	4
1	3	0	0	0	1	0	7	11
2	2	0	0	0	0	0	4	6
3	0	0	0	0	1	0	8	9
4	1	0	0	1	2	1	1	6
5	1	0	0	1	0	0	0	2
6	1	1	0	0	0	0	1	3
7	3	0	0	0	0	0	4	7
8	1	0	0	0	3	0	2	6
9	3	0	1	0	6	2	2	14
10	3	0	0	1	9	0	4	17
Total	19	3	1	3	23	3	33	85

* *PRNP* 132 methionine/methionine.

† *PRNP* 132 methionine/leucine.

‡ *PRNP* 132 leucine/leucine.

§ *PRNP* not determined.

Obex score = 1. Eleven elk had a raw obex score that varied from 1.05 to 1.2. Trace amounts of PrP^{CWD} IR were detected in the lower aspects of the posterior half of the V. Minimal amount of PrP^{CWD} IR was found around the periphery of several neurons and/or within the surrounding neuropil. In 1 elk, PrP^{CWD} IR was exclusively found around several neurons; in 2 elk, PrP^{CWD} IR was only found within the neuropil, and, in 8 elk, PrP^{CWD} IR was found in both locations. PrP^{CWD} IR within the neuropil and on the outer surface of the membranes of neurons was characterized by a coarse red granular material. PrP^{CWD} IR was not found in any of the other nuclei or in axonal tracts. Spongiform degeneration was not present in any of the nuclei.

Obex score = 2. Six elk had a raw obex score that varied from 2.3 to 4.4. Minimal amounts of PrP^{CWD} IR were detected in the V and P in all 6 elk. In 3 elk, PrP^{CWD} IR also was present in the S.

Obex score = 3. Nine elk had a raw obex score that varied from 6.0 to 10.0. PrP^{CWD} IR was detected in the V, P, and S in all 9; A in 4; T in 3; and MR in 2. Plaques of PrP^{CWD} IR were found in the V and S. PrP^{CWD} IR was present in the ST in all 9; RV in 5; and TP in 3. One of these elk also had a trace amount of PrP^{CWD} IR in the SE. A minimal degree of spongiform degeneration was present in V in 3 and in the S in 1 elk.

Obex score = 4. Six elk had a raw obex score that varied from 10.3 to 14.2. PrP^{CWD} IR was detected in the V, P, S, and T in 6; A and MR in 5; C in 3; and O and R in 1 elk. Plaques of PrP^{CWD} IR were found in most of the nuclei. PrP^{CWD} IR was present in the ST in 5; in the TP in 3; RV in 3; and in the SF and SE in 1 elk. A minimal degree of spongiform degeneration was present in the V in 4 of the elk.

Obex score = 5. Two elk had a raw obex score that was 19.15 and 19.7, respectively. PrP^{CWD} IR was detected in the V, P, S, T, A, MR, and H in the elk with the raw score of 19.15. PrP^{CWD} IR also was detected in the C and MR in the elk with the raw score of 19.7. Plaques of PrP^{CWD} IR were found in most nuclei. PrP^{CWD} IR staining was present in the ST, RV, PT, VS, SF, TP, GL, and SE in both elk. A minimal degree of spongiform degeneration was present in the neuropil of the V in the elk with a raw score of 19.15, and a moderate degree of spongiform degeneration was present in the V with minimal spongiform degeneration in the P and S in the elk with a raw score of 19.7. This latter elk did show clinical signs consistent with pneumonia and, at postmortem examination, a severe suppurative bronchopneumonia was found.

Obex score = 6. Three elk had a raw obex score that varied from 23.5 to 24.9. PrP^{CWD} IR was detected in all of the nuclei except the O in 2 elk and the H in 1 elk. Plaques of PrP^{CWD} IR were found in most of the nuclei, and PrP^{CWD} IR was present in all axonal tracts except the MF, TS, and ML. A moderate degree of spongiform degeneration was present in the V with mild spongiform degeneration in the P, S, and T.

Obex score = 7. Seven elk had a raw obex score that varied from 25.6 to 28.8. PrP^{CWD} IR was detected in all of the nuclear areas except for the O in 1 elk. One elk (*PRNP* genotype not determined) had a few neurons within the C in which intraneuronal PrP^{CWD} IR was detected (Fig. 7). Up to this point, intraneuronal PrP^{CWD} IR was not detected. PrP^{CWD} IR was present in all of the axonal tracts. The primary difference between an obex score of 6 and 7 was the slight increase in the overall abundance of PrP^{CWD} IR, and presence of intraneuronal PrP^{CWD} IR. However, the degree of spongiform degeneration was similar.

Table 4. Summary of number of elk, presence or absences of clinical signs observed, locations of PrP^{CWD} immunoreactivity and spongiform degeneration in specific nuclei, and presence of PrP^{CWD} immunoreactivity in axonal tracts in the obex of 85 Rocky Mountain elk (*Cervus elaphus nelsoni*) with naturally occurring chronic wasting disease.*

Obex score	0	1	2	3	4	5	6	7	8	9	10
No. of elk	4	11	6	9	6	2	3	7	6	14	17
Clinical signs observed	No	No	No	No	No	No	No	No	No	Yes	Yes
Nuclei											
Dorsal motor N of vagus		P	P	P/S	P/S	P/S	P/S	P/S	P/S	P/S	P/S
Area postrema			P	P	P	P/S	P/S	P/S	P/S	P/S	P/S
N solitarius			P	P	P	P/S	P/S	P/S	P/S	P/S	P/S
N spinal tract of trigeminal				P	P	P	P/S	P/S	P/S	P/S	P/S
N ambiguus				P	P	P	P/S	P/S	P/S	P/S	P/S
Medial raphe				P	P	P	P	P	P/S	P/S	P/S
Reticular formation					P	P	P	P	P/S	P/S	P/S
Cuneate N					P	P	P	P	P/S	P/S	P/S
Olive N					P	P	P	P	P	P/S	P/S
Hypoglossal N						P	P	P	P	P/S	P/S
Axonal tracts											
Solitary tract				P	P	P	P	P	P	P	P
Radix, vagus nerve				P	P	P	P	P	P	P	P
T area postrema				P	P	P	P	P	P	P	P
Subependyma, 4th ventricle				P	P	P	P	P	P	P	P
Spinal T, fifth nerve					P	P	P	P	P	P	P
Glial limitans						P	P	P	P	P	P
Pyramidal T						P	P	P	P	P	P
Vent spinocerebellar T						P	P	P	P	P	P
Dorsal spinocerebellar T							P	P	P	P	P
Med longitudinal fasciculus								P	P	P	P
Tectospinal T								P	P	P	P
Medial lemniscus								P	P	P	P

* P = PrP^{CWD} immunoreactivity observed; S = spongiform degeneration observed.

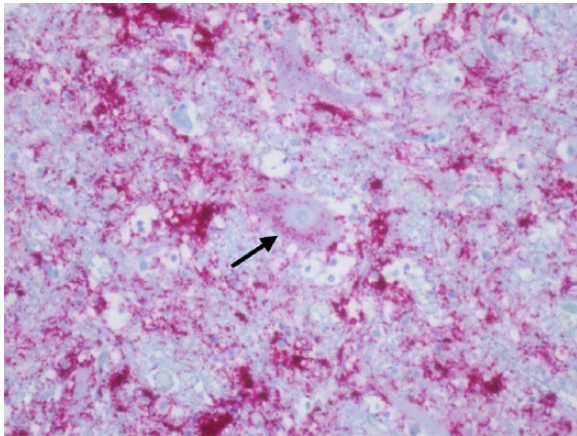


Figure 7. Cuneate nucleus; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 9. Note the intracytoplasmic PrP^{CWD} immunoreactivity within a neuron (arrow), numeric score of 2. Immunohistochemical staining: streptavidin-alkaline phosphatase method. Hematoxylin/bluing counterstain.

Obex score = 8. Six elk had a raw obex score that varied from 30.7 to 34.5. PrP^{CWD} IR was detected in all of the

nuclear areas, and the pattern was similar as to that seen in elk with an obex score of 7. Plaques of PrP^{CWD} IR were present in all nuclei and in all axonal tracts. A moderate to severe spongiform degeneration was found in the V with mild to moderate spongiform degeneration in 5–7 of the other nuclear regions. The primary difference between an obex score of 7 and 8 was an increase in abundance of PrP^{CWD} IR and a slightly greater overall degree of spongiform degeneration.

Obex score = 9. Fourteen elk had a raw obex score that varied from 35.5 to 39.7. PrP^{CWD} IR was detected in all of the nuclear areas with a pattern similar to elk with an obex score of 8, having plaques of PrP^{CWD} IR in all of the nuclei. Ten of the 14 elk had neurons in which intraneuronal PrP^{CWD} IR was detected. A moderate to severe spongiform degeneration was found in the V with mild to moderate spongiform degeneration in 6–9 of the adjacent nuclear regions. The primary difference between an obex score of 8 and 9 was heavier PrP^{CWD} IR and a slightly greater overall degree of spongiform degeneration. Classical clinical signs were first reported in 7 of these elk with an obex score of 9.

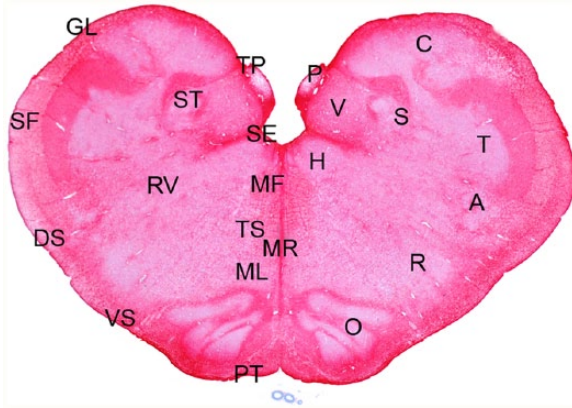


Figure 8. Obex; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 8. This stain demonstrated no difference in the abundance of astrocytes between control elk and elk with different obex scores. This stain also delineated the areas evaluated for scoring. Nuclei (right side): dorsal motor nucleus of the vagus nerve (V), nucleus solitarius (S), area postrema (P), cuneate nucleus (C), nucleus of the spinal tract of the trigeminal nerve (T), nucleus ambiguus (A), reticular formation/nucleus (R), olive nucleus (O), medial raphe (MR), and hypoglossal nucleus (H). Axonal tracts (left side): solitary tract (ST), radix of the vagus nerve (RV), tracts associated with the area postrema (TP), medial longitudinal fasciculus (MF), tectospinal tracts (TS), medial lemniscus (ML), pyramidal tracts (PT), ventral spinocerebellar tracts (VS), dorsal spinocerebellar tracts (DS), and spinal tract of the fifth nerve (SF). Astrocytic layer/glia limitans (GL) and subependyma (SE) of the fourth ventricle. Immunohistochemical staining, glial fibrillary acidic protein: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain, commercial kit.^d

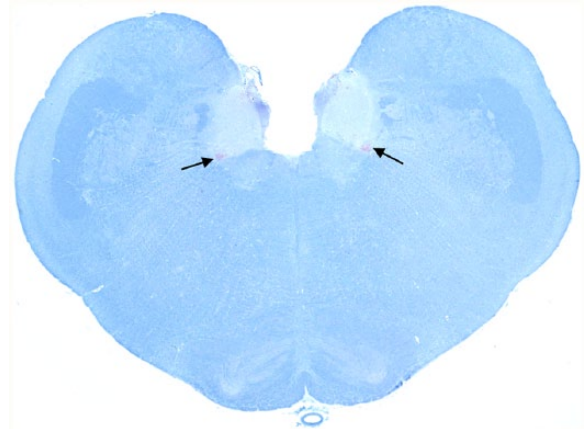


Figure 9. Obex; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132 M/M genotype), obex score = 2. Note the minimal degree of PrP^{CWD} immunoreactivity in the ventral lateral aspects of the dorsal motor nucleus of the vagus nerve (arrows). Immunohistochemical staining: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain.

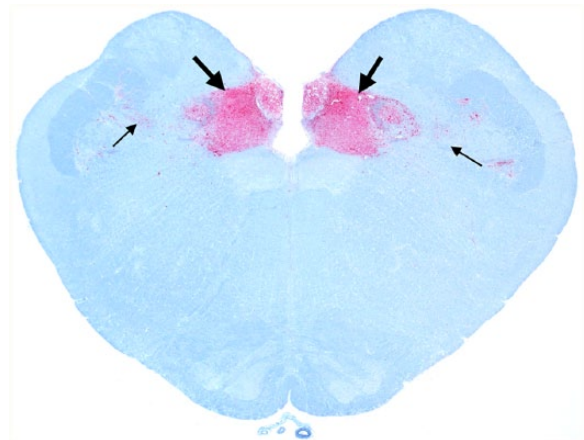


Figure 10. Obex; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 4. Note the intense PrP^{CWD} immunoreactivity (IR) in the dorsal motor nucleus of the vagus nerve and solitary nucleus (thick arrows) and minimal PrP^{CWD} IR in adjacent nuclei (thin arrows). Immunohistochemical staining: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain.

Obex score = 10. Seventeen elk had a raw obex score that varied from 41.1 to 45.7. Moderate to heavy PrP^{CWD} IR was detected in all of the nuclear areas and axonal tracts. The pattern was similar to elk with an obex score of 9 with numerous plaques in all of the nuclei. Intraneuronal PrP^{CWD} IR was detected in all of the 17 elk. Intraneuronal PrP^{CWD} IR was not detected in the V in any of the CWD-positive elk regardless of obex score. Severe spongiform degeneration was found in the DMNV with moderate to severe spongiform degeneration in all of the adjacent nuclear regions. The primary difference between an obex score of 9 and 10 was advanced spongiform degeneration in the nuclear regions. Clinical signs were reported in 12 of 17 of these elk.

The nuclear regions and axonal tracts were well delineated with the GFAP stain for astrocytic processes. The GFAP staining was equivalent in the control and CWD-positive elk, including elk with different obex scores.

Discussion

The pattern of spread of PrP^{CWD} IR and spongiform encephalopathy in a section of obex in elk with naturally occurring CWD progresses in a relatively specific pattern and did not seem to vary with *PRNP* genotype. This spread and increase

in abundance of PrP^{CWD} IR as well as an increase in the severity of spongiform degeneration of the brain stem was remarkable. Clinical signs were not observed in elk with an obex score of 8 or less, and all of these elk were observed prior to euthanasia. Clinical signs were first observed in elk with an obex score of 9. Most of the elk with an obex score of 10 did show clinical signs. Perhaps if the caretakers of the

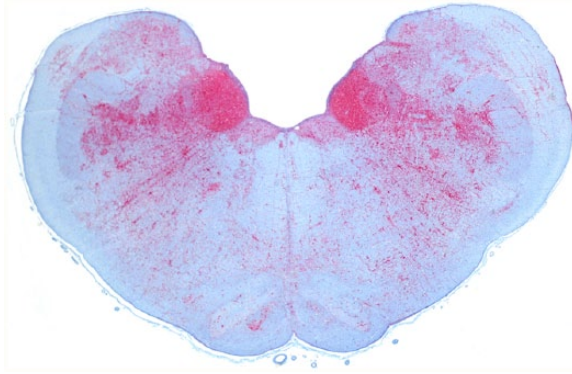


Figure 11. Obex, Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 8. Note the intense PrP^{CWD} immunoreactivity (IR) in the dorsal motor nucleus of the vagus nerve and solitary nucleus and a moderate degree of PrP^{CWD} IR in all of the adjacent nuclei. Immunohistochemical staining: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain.

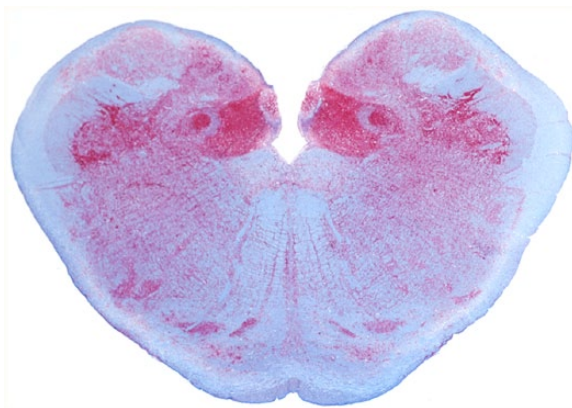


Figure 12. Obex; Rocky Mountain elk (*Cervus elaphus nelsoni*; 132MM genotype), obex score = 10. Note the intense PrP^{CWD} immunoreactivity in all of the nuclei. Immunohistochemical staining: streptavidin–alkaline phosphatase method. Hematoxylin/bluing counterstain.

elk had observed the elk more closely clinical signs would have been observed with earlier obex scores. However, many of these elk were free ranging and were euthanized at random for another project. The reason that clinical signs were not observed earlier no doubt could be associated with the experience of the caretakers; on the other hand, even with domestic sheep, clinical signs are not observed until late in the disease. It is the opinion of the authors that the reason for this finding is that neurons are not widely affected until late in the disease and clinical signs are probably more related to direct neuronal damage than just accumulations of the abnormal conformer of PrP^{CWD} within the neuropil and astrocytes.

Four elk did not have detectable PrP^{CWD} IR in 5 sequential sections of V and palatine tonsil, but did have detectable PrP^{CWD} IR in the medial retropharyngeal lymph node. This

pattern of PrP^{CWD} IR has been reported in a previous study where 43 of 226 (19%) CWD-positive captive elk had staining exclusively in the lymphoid tissues.^{17,20} This pattern of PrP^{CWD} IR is believed to represent early cases of CWD.

PrP^{CWD} IR initially accumulated in the ventral aspects of the middle one-third to posterior half of the V in elk with an obex score of 1. This finding is similar to findings reported in elk,²⁰ free-ranging mule deer,²³ and ranch-raised white-tailed deer.¹¹ Early PrP^{CWD} IR deposition was characterized by coarse granular deposits adjacent to the outer surface of the cell membranes of neurons and/or within the neuropil, and appeared to be associated primarily with synapses and astrocytes. It could not be determined which location was affected first. The significance of this finding is unclear, but may suggest that astrocytes and synaptic junctions are affected and perhaps accumulate PrP^{CWD} before neurons.

The perineuronal PrP^{CWD} IR was characterized by coarse red granular deposits that appeared to be adherent or attached to the outer membrane of perikarya and neurites. This pattern has been described in domestic sheep naturally and experimental infected with scrapie and BSE, and was thought to be associated with astrocytic processes as they attach to cell membranes of the perikarya and neurites.^{3,4,7-9,13-15,24}

Astrocytes have numerous functions within the brain, and have connections to neurons, axons, and vessels. Astrocyte functions include monitoring and regulation of fluids, and electrolytes homeostasis of neurons and surrounding extracellular space, participation in the support of axonal tracts, monitoring and removal of excessive neurotransmitter release at synaptic junctions, protection and insulation of nodes of Ranvier, as well as other functions dealing with the blood brain barrier, healing, and repair.²⁸ Given the specific patterns observed in these early cases of CWD, and comparison with findings in domestic sheep with natural and experimental scrapie and BSE, it was speculated that astrocytes may be one of the first cell types affected by PrP^{CWD} in elk. Surprisingly, the GFAP stain was not useful in detecting differences in the obex scores.

Another possible factor relating to this perineuronal PrP^{CWD} IR could be associated with the multiple synaptic junctions located on membranes of the perikarya called *boutons terminaux*.¹⁰ Abnormal prion protein has been reported to localize in synaptic junctions.⁶ Therefore, this perineuronal pattern maybe associated with PrP^{CWD} accumulation within synaptic junctions located on the membrane of neurons.

PrP^{CWD} IR within axonal tracts was first observed in elk with an obex score of 3 and was consistent and heavily affected in the axon tracts of the S followed by axons from the V. Astrocytic processes can be associated with axons as supporting structures with oligodendritic cells covering axons and nodes of Ranvier.²⁸ The association of astrocytic PrP^{CWD} IR within these axonal tracts may be the route of PrP^{CWD} movement or travel within these tracts and perhaps the brain in general. PrP^{CWD} IR was not observed within the axoplasm of these axons. It was assumed that the PrP^{CWD}

associated with these axons were exiting the brain, but the direction of prion movement could not be determined.

Another pattern of PrP^{CWD} IR was formation of plaques. A few of these plaques had a nucleus in its center that was compatible in size and shape with astrocytic nuclei. These plaques of PrP^{CWD} IR were first observed in the V in elk with an obex score of 2, therefore they formed rather early in the disease. It was speculated that these plaques could be associated, in part, with astrocytes that had their cytoplasm filled with PrP^{CWD}. Plaques also have been associated with astrocytes in domestic sheep with natural and experimental scrapie as well as BSE.^{3,4,7,8,13–15,24} These findings further support early astrocytic involvement with PrP^{CWD} metabolism and perhaps movement within the neuropil.

Intraneuronal PrP^{CWD} IR was not observed until an obex score of 7 was reached. This may suggest that direct neuronal damage does not occur early in the disease. Following the intraneuronal accumulation of PrP^{CWD}, clinical signs shortly followed. The lack of intraneuronal staining in the earlier stages of CWD is probably one reason for lack of clinical signs in these elk. It seemed odd that intraneuronal PrP^{CWD} IR was not observed in the V in any of the elk regardless of obex score.

It has been documented that the amino acid sequence of the *PRNP* gene at codon 132 plays an important role in the duration of the incubation period in elk developing CWD.^{5,17} The 132MM genotype is the most common in free-ranging and captive elk and is associated with an estimated incubation period of ~2 years in experimentally infected elk. The 132ML genotype is not uncommon in captive and free-ranging elk, and the experimental incubation period in elk of this genotype is ~3.5 years.^{5,17} The *PRNP* genotype 132LL are rare (<4%) in free-ranging and captive elk and have an experimental incubation period of ~5–6 years or longer.^{5,17} The ratio of 132MM to 132ML to 132LL of elk in our study was roughly equivalent to the ratio found in free-ranging and captive populations of elk.^{16,18} Differences in patterns of PrP^{CWD} IR deposition were not identified between the various *PRNP* genotypes with regard to progression, abundance, or severity of observed spongiform degeneration. However, the number of times and/or duration of exposure of these elk with the PrP^{CWD} agent were not known. Even though the number of 132ML and 132LL elk with CWD was low, similar patterns of spread of PrP^{CWD} IR and spongiform encephalopathy were observed in this section of obex. It was concluded that the *PRNP* genotypes of the elk did not significantly alter the pattern of progression and abundance of PrP^{CWD} in these nuclei and axonal tracts and the severity of the spongiform degeneration. However, *PRNP* may have an influence on the speed of development of these lesions.

Data from our study suggests that the pattern of CWD progression and abundance of PrP^{CWD} IR in these nuclei and axonal tracts, as well as the progression and severity of the spongiform degeneration have a unique and relatively consistent pattern throughout a section of brain stem at the level

of the obex in Rocky Mountain elk with naturally occurring CWD (Table 4). This technique of scoring the obex may have potential usefulness as a basis for predicting the presence and abundance of PrP^{CWD} in peripheral tissues and the central nervous system.

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Authors' contributions

All authors contributed to conception and design of the study, critically revised the manuscript, and gave final approval. TR Spraker, T Gidlewski, MA Wild, K VerCauteren, and KI O'Rourke contributed to acquisition, analysis, and interpretation of data. JG Powers contributed to analysis and interpretation of data. B Cummings contributed to acquisition of data. TR Spraker drafted the manuscript and agrees to be accountable for all aspects of the work in ensuring that questions relating to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Sources and manufacturers

- Histobond adhesive slides, StatLab Medical Products, McKinney, TX.
- ACROS Organics, Morris Plains, NJ.
- pH 6.1, DAKO target retrieval solution; Dako Denmark A/S, Glostrup, Denmark.
- APK wash solution, NexES automated immunostainer, Basic alkaline phosphatase red kit with amplification, Anti-prion antibody kit; Ventana Medical Systems Inc., Tucson AZ.

Declaration of conflicting interests

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